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Interactions Between Oil Substrates and Glucose on Pure Cultures of Ruminal Lipase-Producing Bacteria

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Abstract The hydrolysis of free fatty acids from lipids is a prerequisite for biohydrogenation, a process that effectively saturates free fatty acids. *Anaerovibrio lipolyticus* 5s and *Butyrivibrio fibrisolvens* have long been thought to be the major contributors to ruminal lipolysis; however, *Propionibacterium avidum* and *acnes* recently have been identified as contributing lipase activity in the rumen. In order to further characterize the lipase activity of these bacterial populations, each was grown with three different lipid substrates, olive oil, corn oil, and flaxseed oil (3 %). Because different finishing rations contain varying levels of glycogen (a source of free glucose) this study also documented the effects of glucose on lipolysis. *P. avidum* and *A. lipolyticus* 5s demonstrated the most rapid rates ($P < 0.05$) of lipolysis for cultures grown with olive oil and flaxseed oil, respectively. *A. lipolyticus, B. fibrisolvens*, and *P. avidum* more effectively hydrolyzed flaxseed oil than olive oil or corn oil, especially in the presence of 0.02 % glucose. Conversely, *P. acnes* hydrolyzed corn oil more readily than olive oil or flaxseed oil and glucose had no effect on lipolytic rate. Thus, these bacterial species demonstrated different specificities for oil substrates and different sensitivities to glucose.

Keywords Lipid chemistry · Lipases · Lipid metabolism · Fatty acid metabolism

Abbreviations
FFA Free fatty acids
CLA Conjugated linoleic acid

Introduction

Diets that contain a high amount of saturated fats have been associated with negative health effects such as increased serum cholesterol levels and risk of coronary heart disease [1–4]. Food products derived from ruminant animals are relatively high in saturated fats, a consequence of microbial biohydrogenation of dietary unsaturated fatty acids. Additionally, greater concentrations of unsaturated fatty acids are positively correlated with the overall palatability of meat [5]. Biohydrogenation is a ruminal process that occurs as a result of microbial metabolic activity [6, 7] which almost quantitatively saturates unsaturated free fatty acids (FFA), thus limiting the availability of unsaturated fatty acids for absorption and assimilation. Biohydrogenation is a detoxification process, necessary for bacteria to escape from the bacteriostatic effects of unsaturated fatty acids [8]. In order for biohydrogenation to occur, FFA must first be hydrolyzed from their triacylglycerol precursors [9]. Conjugated linoleic acid (CLA), a functional fatty acid, is a byproduct of partial fatty acid biohydrogenation [10]. Ruminal isomerization of linoleic acid (18:2 *cis*-9, *cis*-12)

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and desaturation of *trans*-vaccenic acid (18:1 *trans*-11) in tissues of ruminal animals are the only known natural sources of *cis*-9, *trans*-11 CLA [11, 12]. Several efforts have been made to increase the content of CLA in dairy and meat products derived from ruminal animals [13, 14]. Because CLA is a product of biohydrogenation of free fatty acids, lipolysis is essential for the production of CLA.

Anaerovibrio lipolyticus and *B. fibrisolvens* have been identified as the major contributors to overall lipolytic activity in the rumen. *Propionibacterium* species *avidum* and *acnes* also are known to express lipase activity, but little is known about the contribution of these prominent anaerobes to rumen lipolysis. Flaxseed oil, also known as linseed oil, is rich in α-linolenic acid (18:3n-3) which is found predominantly in plants. Weill et al. [15] demonstrated a decreased milk fat percentage in dairy cows fed extruded linseed, but supplementary linseed oil did not affect daily milk fat production.

In order to further characterize the lipase activities of *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. avidum*, and *P. acnes*, each was cultured with three different lipid substrates: olive oil, corn oil and flaxseed oil. Characterizing lipolysis of varying oils in the presence of prominent, ruminal bacteria would aid in the development of a dietary regimen that can lower the impacts of lipolysis and biohydrogenation. Knight and Iliffe [16] reported that glucose increased the accumulation of glycerol in adipose tissue, indicating an increase in lipolysis. High concentrate diets increase the availability of free glucose, so, this study also tested the effects of glucose on hydrolysis of the various lipid substrates. We hypothesize that differences in lipolytic activity will be observed within each bacterium when cultured in varying oil substrates and that such difference will be further affected by free glucose.

Materials and Methods

Treatments

These experiments tested the hypothesis that media glucose would interact differentially with various substrate oils in pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. avidum*, and *P. acnes*. Lipid substrates olive oil, corn oil, and flaxseed oil were added at 3 % of incubation media. Glucose was added at 0.02 % of incubation media.

Pure Bacterial Cultures Used in This Study

Pure cultures of *A. lipolyticus* 5s and *B. fibrisolvens* 49 were obtained from Dr. Jay Yankee, Agriculture-Agri Food Canada. Strains of *P. avidum* and *P. acnes* were previously isolated from the rumen of a pastured cow [17]. For long-term preservation of pure cultures, bacteria were stored in 20 % deoxygenated glycerol at −80 °C. Upon removal from storage, each bacterium was revived via two consecutive 24–48 h cultures in 10 mL standard anaerobic broth supplemented with 2 % pre-sterilized olive oil.

Culture Conditions for Pure Culture Bacteria

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless indicated otherwise. Standard anaerobic broth medium contained (per L): 292 mg K_2HPO_4, 292 mg $KHPO_4$, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4{\cdot}7H_2O$, 64 mg $CaCl_2{\cdot}2H_20$, 4,000 mg Na_2CO_3, 600 mg cysteine HCl, 10 g trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g yeast extract, branched-chain fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methyl butyrate), hemin, vitamin mix (20 mg each thiamine, pantothenate, nicotinamide, pyridoxine HCl, riboflavin, 1 mg *p*-aminobenzoic acid, 0.5 mg biotin, 0.5 mg folic acid, 0.2 mg vitamin B-12, and 0.5 mg lipoic acid) and trace minerals [18]. When glucose was used as a treatment it was added (0.02 %, wt/vol) to a separate batch of media before autoclaving. The medium was further prepared by boiling to remove dissolved O_2 and then saturated with O_2-free gas while cooled on ice under a continuous flow of 100 % CO_2. The cooled medium was distributed (6 mL/tube) using the anaerobic Hungate technique as described by Bryant [19] to 18 × 150 mm glass tubes which were pre-loaded with varying oils (olive oil, corn oil, or flaxseed oil), 3 % (vol/vol). Tubes were closed immediately with rubber stoppers, placed in a press to prevent stoppers from being dislodged, and sterilized by autoclaving. Tubes were cooled to room temperature before inoculation.

Bacteria Growth Rate and Population Evaluation

Growth curves were performed on the pure culture strains to determine the stationary phase of growth of each bacteria in the presence of each substrate. Tubes were inoculated anaerobically with 0.2 mL of each bacterium and incubated at 39 °C in an incubator shaker at 90 rpm. Maximum density was read at 600 nm (A600) every 6 h on a Spectronic 20D+ spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Replicate tubes for each bacterium were inoculated using the same cultures to reduce variation in inoculation density within bacterium. Specific growth rate, μ, was calculated according to the equation ($\mu = \Delta\log_{10}$ A600/Δt, where t = time) [20]. Incubations took place according to conditions described above.

Growth rates were established for each oil substrate in the absence and presence of glucose.

Free Fatty Acid Release During Culture

Tubes were inoculated anaerobically with 0.2 mL of each species of bacterium for comparison of their lipolytic activity in 3 % of varying lipid substrates in the presence or absence of 0.02 % glucose. Tubes were incubated at 39 °C in an incubator shaker at 90 rpm. Concentrated HCl (0.5 mL) was added to stop growth and lipolytic activity following inoculation (zero time controls) and when each bacterium reached a stationary phase of growth. Fatty acids were extracted and quantified by the colorimetric procedures of Kwon and Rhee [21]. The experiment was repeated with the variation that glucose was added to the standard anaerobic medium.

Statistical Analysis

Test for the effects of the different treatments within each bacterium were done using a 2-way general ANOVA (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a Fisher's LSD separation of means ($P < 0.05$). The model included main effects of glucose, lipid substrates, and their interaction; $Y_{ijk} = \mu + \text{glucose}_i + \text{lipid substrate}_j + (\text{glucose} \times \text{lipid substrate})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, glucose and lipid substrate are the fixed effects of the experimental treatments ($i = 1$–2, $j = 1$–3), glucose × oil substrate is their interaction, and ε_{ijk} is the error term.

Results

Effect of Lipids and Glucose on Bacterial Growth Rate

Neither oil substrate types nor glucose affected ($P > 0.05$) the growth rate of *P. avidum* (Table 1). *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P. acnes* had higher growth rates ($P \leq 0.0001$) when glucose was present in the media. *P. acnes* and *A. lipolyticus* had the lowest growth rate when grown in the presence of corn oil, whereas *B. fibrisolvens* 49 had the lowest growth rate in the presence of flaxseed oil. A glucose × lipid interaction ($P < 0.05$) was observed for the growth rate of both *A. lipolyticus* 5s and *B. fibrisolvens* 49. Glucose increased growth rate for *A. lipolyticus* 5s when corn oil was the lipid substrate and increased growth rate for *B. fibrisolvens* 49 when either corn oil or olive oil were the lipid substrates. Glucose had no effect on growth rate for any bacterial species when flaxseed was the oil substrate.

Effect of Lipid and Glucose on Maximum Bacterial Cell Density

Anaerovibrio lipolyticus 5s, *B. fibrisolvens* 49, *and P. acnes* had higher ($P < 0.05$) cell density when glucose was present across all lipid treatments (Table 2). *P. avidum* had a 10 % lower cell density ($P < 0.05$) when glucose was present. *B. fibrisolvens and P. avidum* had higher cell density ($P < 0.05$) when olive oil and flaxseed oil were the oil substrates. A glucose × lipid substrate interaction ($P < 0.05$) was observed for the maximum density of *P. acnes*. Glucose increased the cell density for *P. acnes* when grown in all three lipid substrates, the greatest effect observed when grown in corn oil or flaxseed oil.

Lipolysis

Neither oil substrates nor glucose affected ($P > 0.05$) the accumulation of free fatty acids for *P. avidum* or *P. acnes* (Table 3). Glucose caused a higher accumulation of free fatty acids ($P < 0.05$) when it was present in the media for *B. fibrisolvens*. Glucose did not affect the accumulation of free fatty acids for *A. lipolyticus* or *P. acnes*. Flaxseed oil elicited the highest ($P < 0.05$) rates of FFA accumulation for *A. lipolyticus* 5s and *B. fibrisolvens* 49. There was a significant glucose × lipid interaction for *B. fibrisolvens*. Glucose caused the greatest accumulation of fatty acids when flaxseed oil served as the lipid substrate for *B. fibrisolvens*.

Discussion

Anaerovibrio lipolyticus 5s and some *Butyrivibrio* species play a major role in the ruminal hydrolysis of dietary lipids, with *A. lipolyticus* 5s contributing mainly to the hydrolysis of triacylglycerols and *B. fibrisolvens* contributing mainly but not exclusively to the hydrolysis of galactolipids and phospholipids [22]. More recently, isolations of lipolytic *clostridium*, *Propionibacteria*, *Staphylococcus* and *Selenomonads* from the rumen have been reported [23–26]. *B. fibrisolvens* possesses a broad substrate profile, being able to catabolize and ferment a variety of polysaccharide and protein substrates. Nevertheless, many strains of *B.fibrisolvens,* including strain 49, do not ferment glycerol [27]. Thus, *B. fibrisolvens* 49 likely expresses extracellular lipase to acquire nutritional sources and long chain fatty acids for their cell membranes [28]. Therefore, the stimulation of free fatty acid release from corn or flaxseed oil caused by glucose in *B. fibrisolvens* would enhance the ability of this bacterial species to utilize triacylglycerol fatty acids for energy. *A. lipolyticus* has a broader substrate range, being able to ferment fructose, ribose, lactate and

Table 1 Least square means of the growth rate of ruminal lipase-producing bacteria during growth in the presence of varying lipid substrates with or without added glucose

3 % Lipid substrates (vol/vol)	Mean specific growth rate (μ)							
	Anaerovibrio lipolyticus 5s		*Butyrivibrio fibrisolvens* 49		*Propionibacterium avidum*		*Propionibacterium acnes*	
	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose
Olive oil	0.07^{bc}	0.07^{ab}	0.02^{b}	0.06^{a}	0.07	0.10	0.08	0.09
Corn oil	0.03^{d}	0.08^{a}	0.02^{b}	0.07^{a}	0.05	0.05	0.05	0.07
Flaxseed oil	0.06^{c}	0.07^{abc}	0.03^{b}	0.03^{b}	0.06	0.09	0.08	0.11
SEM	0.006		0.005		0.013		0.005	
P-values								
Glucose	<0.001		<0.001		0.097		0.001	
Lipid	0.036		0.039		0.109		<0.001	
Glucose × lipid	0.001		0.001		0.405		0.344	

^{abcd} Least square means within species with unlike superscripts differ significantly ($P < 0.05$)

Table 2 Least square means of the maximum growth (measured as cell density, A600 nm) achieved by each ruminal lipase-producing bacteria during growth in the presence of varying lipid substrates with or without added glucose

3 % Lipid substrate (vol/vol)	Maximum density (A600 nm)							
	Anaerovibrio lipolyticus 5s		*Butyrivibrio fibrisolvens* 49		*Propionibacterium avidum*		*Propionibacterium acnes*	
	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose
Olive oil	0.95	1.38	1.19	1.37	1.85	1.64	0.89^{c}	1.01^{b}
Corn oil	1.14	1.33	0.10	1.31	1.60	1.46	0.77^{d}	1.18^{a}
Flaxseed oil	1.14	1.23	1.11	1.35	1.68	1.49	0.87^{c}	1.13^{a}
SEM	0.102		0.043		0.048		0.029	
P-values								
Glucose	0.007		<0.001		0.001		<0.001	
Lipid	0.669		0.035		0.002		0.321	
Glucose × lipid	0.183		0.360		0.781		0.001	

^{abcd} Least square means within species with unlike superscripts differ significantly ($P < 0.05$)

Table 3 Least square means for total free fatty acids of cells grown in the presence of varying lipid substrates with 0 and 0.02 % added glucose

3 % Lipid substrate (vol/vol)	Accumulation of free fatty acids (nmol/ml^{-1} h^{-1})							
	Anaerovibrio lipolyticus 5s		*Butyrivibrio fibrisolvens* 49		*Propionibacterium avidum*		*Propionibacterium acnes*	
	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose	0 % glucose	0.02 % glucose
Olive oil	44.2	0.69	92.7^{b}	27.0^{bc}	350.6	609.0	13.3	19.2
Corn oil	44.7	ND	7.7^{c}	85.4^{b}	150.1	353.4	61.1	91.6
Flaxseed oil	70.2	95.2	34.9^{bc}	415.6^{a}	524.8	544.9	9.2	36.5
SEM	22.6		26.9		132.8		25.4	
Glucose	0.207		0.001		0.094		0.961	
Lipid	0.012		0.001		0.092		0.057	
Glucose × lipid	0.152		<0.001		0.371		0.453	

ND not detectable

^{abc} Least square means within species with unlike superscripts differ significantly ($P < 0.05$)

glycerol [29]. Consistent with its broader substrate specificity, *A. lipolyticus* lipolytic rate was independent of glucose.

The present study does not indicate at what point during growth the lipase from these bacteria was produced. Hobson and Summers [30] indicated that *A. lipolyticus* 5s produces two lipases, an esterase associated mainly within the cells and a lipase which is secreted into the culture medium. The secreted lipase was preferentially produced during log phase growth and the esterase during stationary phase.

In batch culture, numerous irregular bulges in the plasma membrane are observed in *A. lipolyticus* 5s during early logarithmic phase of growth, when lipase production appears to be maximal [31, 32]. Hazelwood et al. [28] reported that *B. fibrisolvens* 49 almost completely hydrolyzed galactolipids and phospholipids after 8 h of culturing, suggesting the effective lipases also were produced during log growth. However, very little has been reported on the expression of lipase activity of *B. fibrisolvens* against triacylglycerols.

Pablo et al. [33] reported that *P. acnes* lipase activity was expressed during early log growth, but expression was diminished in older cultures. Holland et al. [34] further investigated lipase production by *P. acnes* and *P. avidum*; their results suggested that the effects of energy source on the expression of lipase activity may be strain-specific. Lipase expression by *P. avidum* was suppressed by glucose, and both glucose and glycerol suppressed lipase production by *P. acnes* strain 37, but neither glucose nor glycerol influenced lipase expression by *P. acnes* strain PF276 [34]. In contrast to the results of Holland et al. [34], in this study glucose actually may have stimulated ($P = 0.09$) free fatty acid release in *P. avidum*.

Both species of *Propionibacterium* differ from *B. fibrisolvens* 49 in that they are able to ferment glycerol [35, 36]. Therefore, these species may hydrolyze triacylglycerols to provide access to glycerol and some of the liberated fatty acids may be assimilated into the lipid membranes of these bacteria. Choi and Song [37] examined the effect of 18-carbon polyunsaturated fatty acids on direct incorporation into rumen bacteria by adding 60 mg of linoleic acid or α-linolenic acid into a rumen fluid, mixed culture solution. The amounts of fatty acids incorporated into the bacteria following a 12-h incubation were 1.20 mg and 0.43 mg/30 mL rumen fluid for linoleic and α-linolenic acid, respectively [37].

Conjugated linoleic acid is an intermediate of ruminal metabolism of dietary linoleic and α-linolenic acid. Conjugated linoleic acid originates from ruminal isomerization, a process that requires lipolysis [10]. It was originally indicated that CLA was derived primarily from linoleic acid in the rumen [38], but Bessa et al. [39] later showed an alternative pathway that derived CLA from α-linolenic acid. Choi and Song [37] supplemented oils rich in either linoleic or α-linolenic acid into mixed ruminal cultures in vitro and found that the production of the CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 was slightly higher in cultures with α-linolenic acid than in those cultured with linoleic acid. Similarly, Wang et al. [40] reported CLA production was greater from sources high in α-linolenic acid than from sources high in linoleic acid. The greatest rates of lipolysis for *A. lipolyticus*, *B. fibrisolvens*, and *P. avidum* were observed when flaxseed oil served as the lipid substrate. This is consistent with Choi and Song [37] and Wang et al. [40].

Cattle fed hay or pasture-based diets, which are relatively rich in α-linolenic acid, have elevated CLA isomers in their tissues, indicating that the α-linolenic is converted to CLA in the rumen [41, 42]. Archibeque et al. [43] reported the fatty acid composition of adipose tissues of steers fed corn- or flaxseed-based finishing diets, in which there was a greater concentration of CLA in adipose tissues of flaxseed-fed cattle than in corn-fed cattle. In the current study, flaxseed was more readily hydrolyzed than corn oil, and thereby could have provided more substrate for the production of CLA than corn oil.

All oil sources contain 10–12 % saturated fatty acids, with varying concentrations of monounsaturated and polyunsaturated fatty acids. Also, the monounsaturated and polyunsaturated fatty acids are distributed in all *sn*-positions, although enrichment at any *sn*-position depends on the oil [44, 45]. Olive oil contains 76 % oleic acid, 14 % linoleic acid, and approximately 1 % α-linolenic acid. The *sn*-2 position of olive oil is particularly enriched with oleic acid (83 % total *sn*-2 fatty acids) and linoleic acid (14 % *sn*-2 fatty acids). Corn oil contains 29 % oleic acid, 57 % linoleic acid, and 1 % α-linolenic acid, again with the unsaturated fatty acids concentrated at the *sn*-2 position. Flaxseed oil contains 16 % oleic acid, 17 % linoleic acid, and 57 % α-linolenic acid. Linoleic acid is concentrated in the *sn*-2 position, whereas α-linolenic acid is more concentrated at the *sn*-2 and *sn*–3 positions (60 % of fatty acids at those positions) than at the *sn*-1 position (53 % of *sn*-1 fatty acids). Results from the present study demonstrated that corn oil exhibits considerable resistance to ruminal lipolysis when compared to flaxseed and olive oil, except in *P. acnes*. These findings suggest that either the fatty acid composition or the *sn*-position distributions of the substrate oils influenced their susceptibility to hydrolysis by these cultures of ruminal bacteria.

A consistent finding of this research is that lipolysis in those bacterial species with the capacity to ferment glycerol (*A. lipolyticus*, *P. avidum*, and *P. acnes*) are refractory to media glucose. Conversely, lipolysis in *B. fibrisolvens,* which cannot ferment glycerol, was profoundly sensitive to glucose when corn oil and flaxseed oil served as the lipid

substrates. The molecular mechanism(s) by which glucose regulates lipolysis warrants further investigation. Additionally, each substrate oil provided a markedly different fatty acid substrate. Therefore future research should document the fatty acid species (to include CLA) that are produced during in vitro incubations with substrate oils that vary widely in fatty acid composition.

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